Biotechnol Lett
DOI 10.1007/s10529-016-2068-5

ORIGINAL RESEARCH PAPER

Improved production of carotenoid-free welan gum in a genetic-engineered *Alcaligenes* sp. ATCC31555

Wenwen Zhang · Zhaohui Chen · Mengmeng Wu · Zhong Shi · Feng Zhu · Guoqiang li · Ting Ma

Received: 6 January 2016 / Accepted: 25 February 2016

Abstract
Objective To improve the production of welan gum and obtain a carotenoid-free strain while reducing the fermentation and post-treatment costs.
Results The vitreoscilla globin (*vgb*) gene combined with the β-galactosidase (lacZ) promoter was inserted into the phytoene synthase (*crtB*) gene region of the chromosome in *Alcaligenes* sp. ATCC31555. When the recombinant strain was grown in a 5 l fermentor, welan gum was produced at 24 ± 0.4 g l^{-1} compared to 21 g ± 0.4 g l^{-1} in the wild type. Furthermore, the carotenoid-free welan gum produced using *Alcaligenes* sp. ATCC31555 VHb strain was less expensive with improved properties.
Conclusions *Alcaligenes* sp. ATCC31555 VHb strain was a better neutral welan-producing strain with a higher production than the wild-type strain.

Keywords *Alcaligenes* sp. · Carotenoid-free strain · Gene expression · Phytoene synthase gene · *Vitreoscilla hemoglobin* · Welan gum

Introduction

Welan gum is an anionic exopolysaccharide produced by *Alcaligenes* sp. ATCC31555 and is commercially available from CP Kelco (Freitas et al. 2011). It is composed of tetrasaccharide-repeating units of D-glucose, D-glucuronic acid, D-glucose, and L-rhamnose, with L-rhamnopyranosyl or L-mannosyl as the side-chains (Chandrasekaran et al. 1994). Welan gum has a stable viscosity in aqueous solutions from 20 to 160 °C and pH from 2 to 12; it has diverse potential applications in food, concrete additives, and drilling mud (Plank 2004). However, the limitation of the productivity of welan gum caused by the O_2 supply remains an important concern in fermentation because

Electronic supplementary material The online version of this article (doi:10.1007/s10529-016-2068-5) contains supplementary material, which is available to authorized users.

W. Zhang · Z. Chen · M. Wu · Z. Shi · F. Zhu · G. li · T. Ma (✉)
Key Laboratory of Molecular Microbiology Technology, Ministry of Education, College of Life Sciences, Nankai University, Tianjin 300071, China
e-mail: tingma@nankai.edu.cn

W. Zhang
e-mail: zww250849@163.com

Z. Chen
e-mail: 13072068533@163.com

M. Wu
e-mail: wumeng6163@163.com

Z. Shi
e-mail: sz@mail.nankai.edu.cn

F. Zhu
e-mail: zhufengsmile@yeah.net

G. li
e-mail: gqli@nankai.edu.cn

Published online: 01 March 2016

the highly viscous broth affects O_2 transfer. During the fermentative production of welan gum, *Alcaligenes* sp. accumulates carotenoid pigments thereby increasing downstream processing costs. It is crucial to increase the efficiency of production and decrease the extraction costs of welan gum to make it a commodity product for large markets.

Vitreoscilla hemoglobin (VHb), synthesized by the Gram-negative bacterium *Vitreoscilla,* enables an organism to survive in a hypoxic environment (Wakabayashi et al. 1986). VHb expression in heterologous bacterial hosts increases the cell density and metabolic production (Kang et al. 2002). We envisioned that the expression of the *Vitreoscilla* globin (*vgb*) gene in a welan-producing strain would alleviate O_2 limitation thus increasing cell growth and welan gum production.

Most *Sphingomonas* species produce carotenoids, identified as nostoxanthin in *Sphingomonas elodea* ATCC31461 (Zhu et al. 2012). To remove the carotenoid pigments in *Alcaligenes* sp., large amounts of ethanol are needed. Thus, a carotenoid-free strain that deactivates carotenoid biosynthesis is necessary for producing neutral welan gum.

The *vgb* gene under the control of a constitutive β-galactosidase (lacZ) promoter was introduced into the *crtB* region of the chromosome in *Alcaligenes* sp. The genetic-engineered *Alcaligenes* sp. VHb strain showed a higher production of welan gum and a lower recovery costs compared to the wild-type strain; it could synthesize welan gum with the same properties as the wild-type strain.

Materials and methods

Strains, culture media, and culture conditions

All the strains and plasmids used are listed in Table 1.

Escherichia coli S17-1 was cultured aerobically in lysogeny broth (LB) with shaking at 200 rpm and 37 °C. TPG medium (pH 7–7.2) contained the following components (l^{-1}): glucose, 15 g; peptone, 5 g; yeast extract, 3 g; beef powder, 3 g. The seed medium (pH 7–7.2) was prepared from the following components (l^{-1}): glucose, 15 g; peptone, 2.5 g; yeast extract, 1.5 g; K_2HPO_4, 2.5 g; $(NH_4)_2HPO_4$, 1.5 g; and $MgSO_4$, 1 g. The fermentation medium (pH 7–7.2) contained the following components (l^{-1}): glucose, 50 g; corn starch, 1 g; $NaNO_3$, 4 g; $Na_2HPO_4{\cdot}7H_2O$, 9.28 g; $MgSO_4$, 0.8 g; and $CaCO_3$, 4.65 g.

The inoculum was grown in a seed medium at 30 °C and 200 rpm for 14 h. The fermentations were carried out with a 10 % (v/v) inoculum into 500 ml Erlenmeyer flasks at 30 °C and shaking at 200 rpm for 72 h.

The batch fermentations were studied in a 5 l autoclavable fermentor containing 3.5 l medium. The process set points were 30 °C; pH, 7 ± 0.1; agitation rate, the first 24 h, 400 rpm, and the next 48 h, 600 rpm; and aeration rate, 1 vvm. The pH was controlled by an automatic addition of 1 M NaOH or 1 M HCl. Antibiotics were used as follows: 100 mg ampicillin l^{-1}, 34 mg kanamycin l^{-1}, and 10 mg tetracycline l^{-1}.

DNA manipulation and construction of mutant strains

Primers used are listed in Supplementary Table 1. Suicide vector pLO3 was used to construct the *crtB* gene deletion vector, pLO3-ΔcrtB. The upstream and downstream flanking sequences of *crtB* gene were amplified with PrimeSTAR DNA Polymerase (Takara Bio, Tokyo, Japan) using primers crtB-SF/crtB-SR and crtB-XF/crtB-XR, respectively. The two regions of the DNA fragments were joined by overlap PCR. The generated fragment was restricted by *Sal*I and *Xba*I, and cloned into suicide vector pLO3 using the same enzymes, affording the deletion plasmid pLO3-ΔcrtB. This was then transformed into *E. coli* S17-1.

Suicide vector pLO3 was used to construct the *vgb* gene insertion vector, pLO3-vgb. To construct pBBR-vgb, plasmid pKSPVK was used to amplify the *vgb* gene sequence and cloned into pBBR1MCS2. Then, PlacZ-vgb sequence was amplified from pBBR-vgb using primers Pvgb-F/Pvgb-R. The upstream and downstream flanking sequences of *crtB* gene were amplified with PrimeSTAR DNA Polymerase using primers crtB-SF/crtB-XSR and crtB-XXF/crtB-XR, respectively. These three regions of DNA fragments were joined by overlap PCR. Then, the generated fragment was restricted by *Sal*I and ***Xba***I, and cloned into suicide vector pLO3 using the same enzymes, affording insertion plasmid pLO3-vgb. This was then transformed into *E. coli* S17-1. A gene marker-less knockout method was used to construct the insertion mutant based on homologous recombination (Fig. 1b).

Table 1 Strains and plasmids

Strains and plasmids	Relevant genotype and characteristics	References
Strains		
E. coli s17	recA pro hsdR RP4-2-Tc::Mu-Km::Tn7	This lab
Alcaligenes sp. ATCC 31555	wild-type strain; Amp^r	This work
Alcaligenes sp. Δ*crt*B	derivative, Δ*crt*B	This work
Alcaligenes sp. VHb	derivative, Δ*crt*B, lacZ-*vgb*	This work
Plasmids		
pLO3	sacB; Tc^r; oriT	Lenz and Friedrich (1998)
pLO3-ΔcrtB	pLO3—derivation with deletion fragment of *crt*B	This work
pLO3-vgb	pLO3—derivation with insertion fragment of PlacZ-*vgb*	This work
pBBR1MCS2	oriT; Km^r	Kovach et al. (1995)
pKSPVK	pKSV7 derivative consists of promoter p43, *vgb*	Zhang et al. (2013)

Reverse transcription PCR (RT-PCR) analyses

Purezol reagent combined with RNAprep pure Cell/Bacteria Kit was used to extract the total RNA. The residual genomic DNA contamination was removed by on-column DNase digestion. The cDNA was obtained by reverse transcriptional synthesis using a Quantscript RT Kit.

Analytical methods

The welan gum concentration and dry cell weight (DCW) were measured as previously reported (Ai et al. 2015). The fermentation broth viscosity was measured using a viscometer lV-DV_II+ equipped with a rotor (No. 64) at 60 rpm. The pH was measured using the inductor of the bioreactor. The glucose content was measured using a SBA-40C biosensor equipped with a glucose oxidase electrode. All the values are the mean of three cultures, replicated three times.

Results and discussion

Identification of the *crtZ*, *crtB*, *crtI*, *crtY*, and *crtG* genes of *Alcaligenes* sp. ATCC 31555

Alcaligenes sp. synthesizes a carotenoid in the product. Zhu et al. (2012) characterized the carotenoid biosynthesis genes in the gellan gum-producing bacterium *Sphingomonas elodea* ATCC 31461 containing *crtZ*, *crtB*, *crtI*, *crtY*, and *crtG*. The five putative carotenogenic genes were found in *Alcaligenes* sp. ATCC 31555 using the National Center for Biotechnology Information (NCBI) Blast program (Blastp; Fig. 1a).

Compared to the yellow-pigmented wild-type strain, the ΔcrtB mutants were colorless (Fig. 1c) and lacked phytoene synthesis, indicating that the knockout of *crtB* encoded with the predicted phytoene synthase (accession number WP_019370947.1) was responsible for the loss of pigment in the ΔcrtB mutants. To evaluate and compare the welan gum concentration of the ΔcrtB mutants with the wild-type strain, fermentation experiments were carried out in 250 ml flasks. Overall, the broth viscosity and welan gum concentration of the ΔcrtB mutants and wild-type strain were almost identical, 4120 ± 71.7 mPa s and 18.4 ± 0.4 g l^{-1}, respectively. Moreover, it was economic to recover welan gum from the colorless fermentation broth. This is because a yellow fermentation broth requires more than three volumes of ethanol to remove the pigments closely bound to the welan gum.

Identification of *crtB* gene knockout and *vgb* gene insertion

To construct a genetic-engineered strain of *Alcaligenes* sp., the *vgb* gene combined with the lacZ promoter was inserted into the *crtB* gene region of the

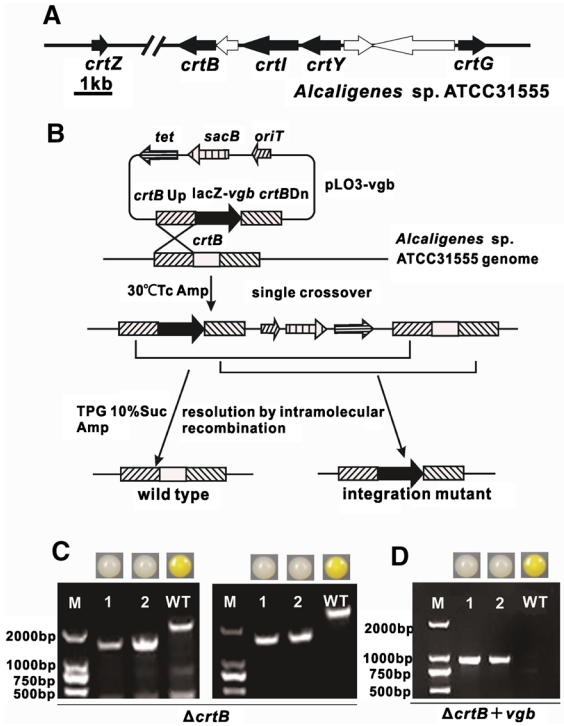

Fig. 1 **a** Carotenogenic gene cluster in *Alcaligenes* sp. **b** Scheme of the recombination events leading to gene insertion. pLO3-vgb was transferred to *Alcaligenes* sp. via triparental conjugation. The single-crossover recombinants were obtained by spreading the transformants on TPG plates with Amp^r and Tc^r pressure. Selected single-crossover recombinants were then incubated in the seed medium and spread on the TPG plates containing 10 % sucrose to facilitate intramolecular recombination, affording the desired mutants. The chromosome structures of the final mutants were confirmed by PCR. Note that the initial recombination can occur either in the upstream or downstream portion of the target gene as shown here. In both the cases, the final structure of the chromosome is the same. **c** Confirmation of the *crtB* deletion by PCR using two pairs of primers. Chromosomal DNA served as the template for amplification. lane M, DNA marker; lanes 1 and 2, gene deletion strains amplified with primers crtB-1S/crtB-1X (*left*) and crtB-2S/crtB-2X (*right*), respectively; lane WT, wild-type strain amplified with relative primers. **d** Confirmation of the *vgb* insertion by PCR. lane M, DNA marker; lanes 1 and 2, gene insertion strains amplified with primers Pvgb-F/Pvgb-R; lane WT, wild-type strain amplified with relative primers

chromosome by suicide vector pLO3-vgb (Fig. 1b). The *crtB* encoded with the predicted phytoene synthase was inactivated by the *vgb* insertion; the mutants were also colorless (Fig. 1d). The results indicate that the genetic-engineered strain inhibited phytoene synthesis by *vgb* insertion.

Confirmation of the expression of the *vgb* gene and its effect under different aeration conditions

The RT-PCR and CO difference spectra were used to further confirm the expression of *vgb* gene. The *vgb* gene transcriptional levels of *Alcaligenes* sp. VHb strain were detected (Supplementary Fig. 1a). The VHb binding with CO shows a characteristic absorption peak at 420 nm (Liu and Webster 1974). The CO difference spectra (Supplementary Fig. 1b) clearly showed a pronounced absorption peak at ~420 nm. In contrast, this peak was absent in the wild-type strain. The results indicate that VHb synthesized by the engineering strain is biologically active.

VHb expression enhances cell growth and metabolite production (Zhang et al. 2013). To evaluate the effects of VHb on welan gum concentration and viscosity, the wild-type and *Alcaligenes* sp. VHb strains were grown in 500 ml Erlenmeyer flasks under different aeration conditions for 72 h; significant differences in the welan gum concentration and broth viscosity were observed between the strains. Table 2 shows that *Alcaligenes* sp. VHb strain exhibited ~11.7 % higher production than the wild-type strain under strong aeration. The effect of VHb expressed under middle aeration was between those under high and low aerations. As expected, *Alcaligenes* sp. VHb strain under a low aeration exhibited the largest advantage over the wild-type strain, leading to a 25 % higher yield. The broth viscosity showed almost the same trend as the yield of welan gum. This indicates that the function of VHb may vary under different growth conditions, consistent with a previous report by Wu et al. (2010). The gellan gum yields by pCPP30-VHb-bearing strain were 17.4 and 26.8 % higher than those by the wild-type control when cultivated under high and low aeration conditions, respectively. However, the VHb-expressing plasmid may be unstable in *S. elodea*. Therefore, the *vgb* gene under the control of a strong promoter was introduced into the chromosome of *Sphingomonas* to solve the increasing demand of aerobic process in polysaccharide production.

Cell growth, substrate consumption, welan gum concentration, and viscosity in batch cultivation

Alcaligenes sp. VHb strain showed a higher welan gum concentration and viscosity than the wild-type

Table 2 Residual glucose, broth viscosity (mPa s), and welan gum concentration (g l^{-1}) of *Alcaligenes* sp. VHb and wild-type strain under different aeration conditions in Erlenmeyer flasks

Low aeration				Moderate aeration			High aeration			
Strain	Residual glucose	Broth viscosity	Welan production	Residual glucose	Broth viscosity	Welan production	Residual glucose	Broth viscosity	Welan production
VHb	12 ± 0.3	2959 ± 64.1	10.6 ± 0.3	10 ± 0.2	4489 ± 87	15.6 ± 0.2	8.9 ± 0.1	5125 ± 113.1	21 ± 0.3
WT	13.1 ± 0.2	2290 ± 48.3	8.5 ± 0.2	11.4 ± 0.2	3699 ± 70.7	13 ± 0.2	10.1 ± 0.2	4394 ± 90.5	18.8 ± 0.4
F (%)	−8.4	29.2	24.7	−12.3	21.4	20	−11.9	16.6	11.7

All the values are the mean of three cultures, replicated three times

VHb Alcaligenes sp. VHb, *WT* wild type, *F* (VHb−WT)/WT × 100 %

strain throughout the fermentation process (Fig. 2). After 72 h of cultivation, the concentration of welan gum produced by the genetic-engineered *Alcaligenes* sp. VHb strain was up to 24 ± 0.4 g l^{-1}, 14 % higher than that produced by the wild-type strain. Such an increase was also observed for the viscosity of the fermentation broth. The viscosity of the fermentation broth for the VHb and wild-type strains increased to a maximum of 5980 ± 117.8 and 6799 ± 125.8 mPa s, respectively. The DCW of *Alcaligenes* sp. VHb strain was higher than that of the wild-type strain throughout the cultivation. Furthermore, the glucose consumption rate was higher for *Alcaligenes* sp. VHb strain than for the wild-type strain over the same period of fermentation. The higher glucose consumption rate of *Alcaligenes* sp. VHb strain can be attributed to a faster rate of welan production. At the end of fermentation, the glucose conversion rates were 48 and 42.2 % for *Alcaligenes* sp. VHb strain and the wild-type strain, respectively.

Many attempts have been made to improve welan gum production, including agitation control, use of additives, and process optimization (Ai et al. 2015). However, a few metabolic engineering strategies have been reported to improve welan gum production. In a previous study, the dissolved O_2 concentration increased by adding an O_2 vector or agitation control, incurring additional cost. VHb interacts with the terminal respiratory oxidase by delivering O_2 to it, increasing oxidative phosphorylation and thus the production of adenosine triphosphate (Park et al. 2002). Thus, *Alcaligenes* sp. VHb strain could produce welan gum without additional costs. The concentration of welan gum produced by *Alcaligenes* sp. VHb strain was 24 ± 0.4 g l^{-1}, whereas those produced by *Alcaligenes* sp. NX-3 (Li et al. 2010a, b) and *Alcaligenes* sp. NX-3-1(Li et al. 2010) were 22.9 and 21.8 ± 0.24 g l^{-1}, respectively. Therefore, *Alcaligenes* sp. VHb strain is a better welan-producing strain with a higher production than the wild-type strain.

Recovery and properties of welan gum

Another significant advantage of *Alcaligenes* sp. VHb strain for welan production is that it is non-pigmented. The residual carotenoid pigments make the product a dark yellow. When a pure welan product is desired, the key step is the removal of the pigments (major impurities). Excess ethanol must be used to remove

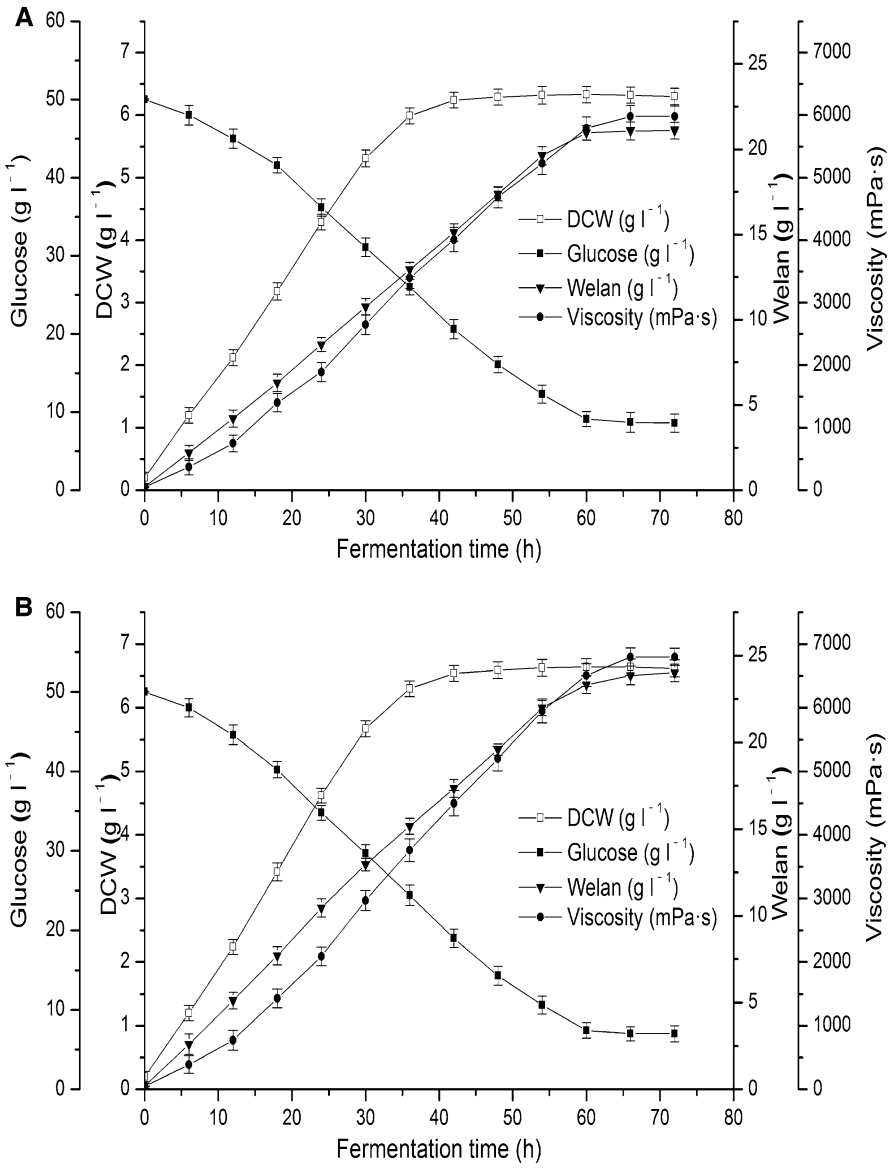

Fig. 2 Fermentation profile of the wild-type strain (**a**) and *Alcaligenes* sp. VHb strain (**b**) in 5 l bioreactors for 72 h. The cells were grown under the same fermentation conditions (*error bars* indicate SD)

the pigment impurities, increasing the total cost. In contrast, three volumes of ethanol were sufficient for the welan gum recovery from *Alcaligenes* sp. VHb strain. In the production of welan using a wild-type strain, it was difficult to reduce the cost. Thus, the decrease in the pigment content in the genetic-engineered strain provides a more economic down-stream purification process.

The molecular weight and polydispersity index of welan gum secreted by *Alcaligenes* sp. VHb strain and wild-type strain were 600 kDa and 1, respectively. To further characterize the properties of welan produced using the engineered strain, the apparent viscosity of the welan gum from *Alcaligenes* sp. VHb strain was compared to that of the wild-type strain (Fig. 3a). The apparent viscosity of a solution of 0.2–1 % (w/v) welan gum produced by the strains was measured; no difference was observed in both the strains. We also investigated the temperature dependence of the rheological properties of welan gum produced by both the strains. As shown in Fig. 3b, the viscosity decreased slightly above 80 °C; thus, the temperature slightly affected the apparent viscosity of the two welan gum solutions. This indicates a good thermal stability of the

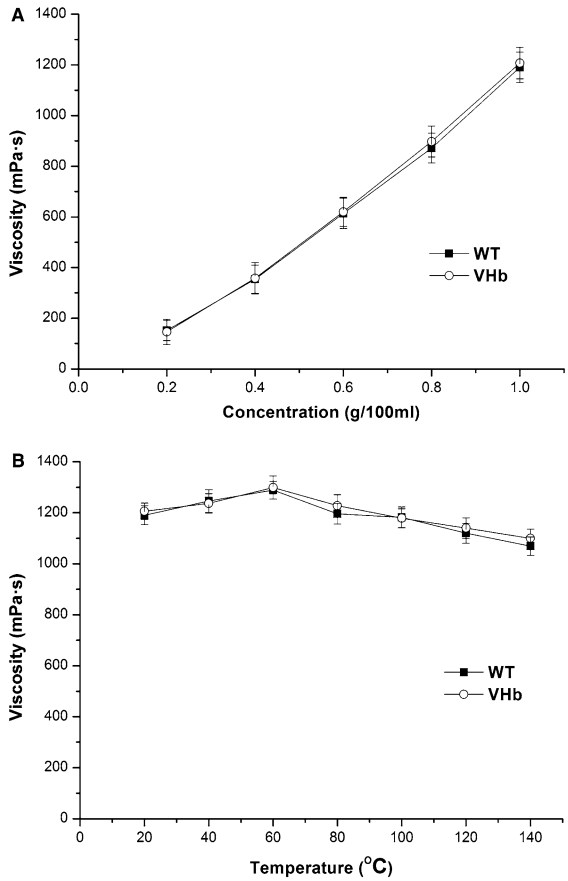

Fig. 3 Rheological properties of the solutions of welan gum produced by wild-type and *Alcaligenes* sp. VHb strains. The experiments were carried out at 25 °C equipped with a rotor (No. 63) at 60 rpm. **a** The apparent viscosity of solutions of 0.2–1 % (w/v) welan gum. **b** Effects of temperature on the viscosity of welan gum (1 % solution). All the values are the mean of three cultures, replicated three times (*error bars* indicate SD)

two welan gum solutions, consistent with the previous report that welan gum solutions maintain a high viscosity even at a high temperature (Ai et al. 2015). Because welan gum retains its stability and viscosity at a high temperature, it has potential applications in oil-well drilling.

Acknowledgments This work was supported by grants from the National Natural Science Foundation of China [grant numbers 31571790, 41373074].

Supporting Information Supplementary Table 1—Primers used.

Supplemenmtary Fig. 1—Conformation of the expression of *vgb* gene.

References

Ai H, Liu M, Yu P, Zhang S, Suo Y, Luo P, Li S, Wang J (2015) Improved welan gum production by *Alcaligenes* sp. ATCC31555 from pretreated cane molasses. Carbohydr Polym 129:35–43

Chandrasekaran R, Radha A, Lee EJ (1994) Structural roles of calciumions and side chains in welan: an X-ray study. Carbohydr Res 252:183–207

Freitas F, Alves VD, Reis MAM (2011) Advances in bacterial exopolysaccharides: from production to biotechnological applications. Trends Biotechnol 29(8):388–398

Kang DG, Kim JYH, Cha HJ (2002) Enhanced detoxification of organophosphates using recombinant *Escherichia coli* with co-expression of organophosphorus hydrolase and bacterial hemoglobin. Biotechnol Lett 24:879–883

Kovach ME, Elzer PH, Hill DS et al (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS2, carrying different antibiotic-resistance cassettes. Gene 166:175–176

Lenz O, Friedrich B (1998) A novel multicomponent regulatory system mediates H2 sensing in *Alcaligenes eutrophus*. Proc Natl Acad Sci USA 95:12474–12479

Li H, Xu H, Li S et al (2010a) Strain improvement and metabolic flux modeling of wild-type and mutant *Alcaligenes* sp. NX-3 for synthesis of exopolysaccharide welan gum. Biotechnol Bioprocess Eng 15:777–784

Li S, Xu H, Li H, Guo C (2010b) Optimizing the production of welan gum by *Alcaligenes facalis* NX-3 using statistical experiment design. Afr J Biotech 9:1024–1030

Liu CY, Webster DA (1974) Spectral characteristics and interconversions of the reduced, oxidized, and oxygenated forms of purified cytochrome o. J Biol Chem 249: 4261–4266

Park KW, Kim KJ, Howard AJ, Stark BC, Webster DA (2002) Vitreoscilla hemoglobin binds to subunit I of cytochrome bo ubiquinol oxidases. J Biol Chem 277:33334–33337

Plank J (2004) Applications of biopolymers and other biotechnological products in building materials. Appl Microbiol Biotechnol 66:1–9

Wakabayashi S, Matsubara H, Webster DA (1986) Primary sequence of a dimeric bacterial haemoglobin from *Vitreoscilla*. Nature 322:481–483

Wu XC, Chen YM, Li YD et al (2010) Constitutive expression of *Vitreoscilla haemoglobin* in *Sphingomonas elodea* to improve gellan gum production. J Appl Microbiol 110: 422–430

Zhang W, Xie H, He Y et al (2013) Chromosome integration of the *Vitreoscilla hemoglobin* gene (*vgb*) mediated by temperature sensitive plasmid enhances γ-PGA production in *Bacillus amyloliquefaciens*. FEMS Microbiol Lett 343: 127–134

Zhu I, Wu X, Li O, Qian C, Gao H (2012) Cloning and characterization of genes involved in nostoxanthin biosynthesis of *Sphingomonas elodea* ATCC31461. PLoS ONE 7(4): e35099